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RECONSIDERATION OF THE TAXONOMIC STATUS OF MASON'S LILAEOPSIS – A STATE-PROTECTED RARE SPECIES IN CALIFORNIA

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Abstract

Lilaeopsis masonii is a California state-listed rare species with a wide range of morphologies observed in the field throughout its range, and in herbaria collections. This extensive variation confounds reliable taxonomic identification, particularly for those specimens intermediate between *L. masonii* and its sister taxon, *L. occidentalis*. To investigate the genetic basis of this morphological variation, we examined two portions of the *Lilaeopsis* genome in seven species. Specifically we sought to determine whether *L. masonii* is sufficiently distinct from its closely related, widespread congener to continue to warrant specific status. DNA sequence analysis of ITS1, 5.8S, and ITS2 nuclear ribosomal DNA revealed no differences between *L. occidentalis* and *L. masonii* California collections, and minimal differences between these samples and *L. occidentalis* collected from the state of Washington, suggesting strongly that these two species form a single clade. A combination of fragment data from three AFLP primers yielded 274 fragments from 29 samples. Genetic Manhattan distance values calculated from the AFLP matrix within species ranged from a low of 1.4 to a high of 6.6, reflecting minor differences among all samples. UPGMA cluster phenograms support the results of the PCA analysis, illustrating a cluster of *L. occidentalis* + *masonii* samples distinct from other *Lilaeopsis* species. Because conservation dollars should protect unique evolutionary entities, we suggest that *L. masonii* be subsumed under *L. occidentalis* and therefore no longer receive formal state protection.

Key Words: AFLP, Apiaceae, California endangered species Act, goldilocks conundrum, ITS, *lilaeopsis masonii*, *lilaeopsis occidentalis*, UPGMA.

Lilaeopsis masonii Mathias & Constance (Mason's lilaeopsis) is one of 15 wetland or aquatic species of the widespread genus *Lilaeopsis* Greene within the Apiaceae. The genus *Lilaeopsis* is comprised of perennial herbs characterized by a horizontal stem with leaves commonly in clusters ("ramets") borne directly from the stem, although rarely leaves occur individually. *Lilaeopsis* is notable in its morphologic simplicity—entire, generally linear leaves; simple umbels; absence of a carpophore; and, a strongly reduced habit (Petersen et al. 2002; Downie et al. 2000; Downie et al. 2008). Such simple morphology has led to a long history of taxonomic uncertainties and difficulty in the reconstruction of its phylogeny.

Evidence for monophyly of *Lilaeopsis* is strong (Petersen et al. 2002). However, recent research based on molecular evidence from nuclear and chloroplast genes suggests that the genus is best placed in the Oenantheae tribe within the Apioideae (Downie et al. 2008) and that *Lilaeopsis* is sister to the clade comprising *Ptilimnium, Limnosciadium, Daucosma, Cynosciadium* and rachis-leaved species of *Oxypolis*, not the Mexican genus *Neogoezia* as suggested by Petersen et al. (2002). The New World endemics clade of tribe Oenantheae is native to North America and comprises a monophyletic group that appears to be evolving much faster than any other major clade recognized in the tribe (Hardway et al. 2004).

Early taxonomic work on the genus in California by Hill (1927) and Mason (1957) included mention of comparatively smaller and narrower leaves in *Lilaeopsis* specimens occurring away from the coast, in contrast to a relatively more robust coastal form. Professor Herbert Mason, an early expert on the wetland flora of California, first collected a relatively smaller *Lilaeopsis* from Brannan Island of the San Francisco Bay/Sacramento-San Joaquin Delta (Bay-Delta). He referred to the smaller form as the "San Francisco Bay and river-mouth" form (Mason 1957: 631). This specimen, according to Mason (unpublished) was "definitely distinct from the coastal *L. occidentalis*." Western lilaeopsis (*Lilaeopsis occidentalis* J. M. Coulter & Rose) is a widespread, common species,

ranging from the Queen Charlotte Islands of British Columbia, Canada to Marin County, California (Affolter 1985). Considered to be a coastal species confined to salt water or brackish water intertidal habitats, collections of *L. occidentalis* from inland fresh water lentic and lotic habitats are known, but considered "uncharacteristic" (Affolter 1985).

Lilaeopsis masonii was not described as a distinct taxon for two decades after the smaller form in the Bay-Delta was first observed. In 1977, Mathias and Constance formally recognized the diminutive nature of a specimen obtained from Twitchell Island in the Bay-Delta as *L. masonii* (Mathias and Constance 1977). Mathias and Constance described *L. masonii* as distinct from *L. occidentalis* based upon the former (rare) taxon bearing narrower, typically shorter, and more or less terete leaves, and an inland distribution. They honored Herbert Mason's expertise in the wetland flora of the State with the specific epithet.

Mason's lilaeopsis was one of the first vascular plant species to be protected as "rare" under the California Endangered Species Act (CESA) (California Fish & Game Code §§2050, *et seq.*). At the time of its listing in November 1979, only seven population occurrences were known (CNDDB 2009). Since formal protection, the documented extent of geographic distribution and population abundance of *L. masonii* has increased nearly three-fold, primarily as a result of concentrated field survey efforts conducted in the early 1990's by Golden, Fiedler, and Zebell (Golden and Fiedler 1991; Golden 1992; Fiedler and Zebell 1993; Zebell and Fiedler 1996). Today, Mason's lilaeopsis is known to occur within 24 USGS quadrangles and seven counties (CNPS 2008), spanning across roughly 690 square miles. One hundred eighty-six documented occurrences are on record with the state (CNDDB 2009), although most, but not all are extant.

A History of Taxonomic Uncertainty

Confusion over the taxonomic limits of this rare species existed from the beginning of its description. Two examples are relevant. First, a long-controversial *Lilaeopsis* specimen collected by Schreiber (#2266 UC, 28 June 1936) from Chicken Ranch Beach in Marin County derives from outside the circumscribed geographic range of the endemic inland taxon. Leaf lengths from this specimen range between 15 to 42 mm, a morphological range characteristic of comparatively larger leaf lengths for *L. masonii*. However, it is possible to key these larger leaved Chicken Ranch Beach specimens to *L. occidentalis* in every relevant flora (e.g., Hickman 1993).

Affolter (1985) examined this specimen in his monograph of *Lilaeopsis,* and accepted it as *L. masonii*, but noted that it was a geographical outlier for the rare, Bay-Delta endemic species. Today, CNPS (Tibor 2001) acknowledges that this specimen is likely to be *L. occidentalis*, not the rare *L. masonii*, but provides no explanation. Several attempts to relocate this *Lilaeopsis* material at Chicken Ranch Beach by the authors have failed as the population appears to be extirpated, thereby making an independent species corroboration impossible.

It is important to note that (1) numerous collections of *L. occidentalis* from the beaches of Marin and Sonoma counties exist, (2) leaf lengths range by an order of magnitude or more within and between adjacent populations of *L. occidentalis*, (3) the number and clarity of internal crosswalls considered important diagnostic characters are more likely a function of relative plant size, exposure, or both, and (4) inland collections of the common species are known from the state of Washington (e.g., UC 1594452; 4 September 1962). Also noteworthy, *L. masonii* has never again been collected on the Pacific coast of North America beyond Schreiber's Marin Co. collection in 1936.

Further, Affolter (1985) remarked that leaves from a collection of *L. masonii* (derived from Sherman Island immediately down river of Twitchell and Brannan islands) cultivated for his greenhouse comparisons were "remarkably longer than any of the herbarium material" (Affolter 1985:70). He suggested the observed overall larger and more robust greenhouse material was evidence of how difficult it is to understand vegetative plasticity from herbarium material alone. However, the relatively robust response of Mason's lilaeopsis to the mild conditions of a greenhouse suggests strongly that strict morphological distinctions between the two taxa are problematic.

Additional morphological characters further support the assertion that *L. masonii* is not distinctly different from *L. occidentalis*. Affolter (1985:70) noted that the "two taxa are similar in several respects," including similar (1) leaf shapes (linear), (2) rhizome branching architecture, (3) fruit shapes, (4) fruit cell types, (5) fruit venation patterns, (6) habitats, and they have (7) overlapping geographic distributions. Despite all these similarities, Affolter (1985:71) supported their separate specific status, primarily because "when grown under a common-garden environment in the greenhouse, the two species retained the vegetative characteristics that distinguish them in the field."

Subsequent laboratory studies conducted by the principal author and her students (Golden and Fiedler 1991; Golden 1992; Fiedler and Zebell 1993; Zebell and Fiedler 1996) have provided little clarity. Most importantly, no nucleotide variation was found among nine

populations of *L. masonii* or between *L. occidentalis* and *L. masonii* when the 204 nucleotides of the ITS2 nuclear genome were ascertained (Fiedler and Zebell 1993). Fiedler and her colleagues thus concluded tentatively that the rare species was most likely an inland ecotype not clearly distinct from its widespread congener.

Field Observations

Decades of field observations of *Lilaeopsis* throughout the Bay-Delta, Suisun Marsh, and Napa River ecosystems do not reinforce many of the conclusions offered by Hill (1927), Mason (1957, unpublished), Mathias and Constance (1977), and Affolter (1985) supporting the recognition of two distinct taxa. Rather, the few vegetative characteristics that typify this genus are highly variable both within and between populations throughout this region. Occurrences of *L. masonii* in the lower Napa River, studied since 2001 (WSP 2007, unpublished; Blasland, Bouck, & L, Inc. unpublished; Entrix unpublished; L.C. Lee & Associates unpublished; Stillwater Sciences and Fiedler unpublished) include a full spectrum of individual ramet sizes. Often, both large and small forms of *Lilaeopsis* species, easily identifiable to the two different species, can be found growing in the same location. Often the plant stature/leaf length size gradient runs perpendicular to the shoreline, where the small "*masonii*" form (approx.1.5–4.5 cm in height) grows relatively close to the water's edge, while increasing larger and more robust "*occidentalis*" (approx. $\geq$11 cm in height) can be found further from the water. "Intermediate" or medium-sized *Lilaeopsis* material (approx. >6.25 and <11 cm in height) is common throughout this shoreline/river bank habitat and geographic range, and keys to either (or both) the rare or the common species. We call this phenomenon—i.e., range in size of a critical morphological character, with significant overlap between taxa—the "Goldilocks Conundrum" to highlight the problem that the intermediate-sized material is not "just right," but rather, highly problematic.

To resolve our conundrum and determine whether *L. masonii* is a discrete species distinct from *L. occidentalis*, we initiated a genetic analysis of seven species of this genus. We hypothesized that there were no significant differences between diagnostic portions of the genome selected for this study of the two species, *L. masonii* and *L. occidentalis*. Based on these analyses, we then explored whether *L. masonii* warrants continued recognition as a distinct species or rather, should be subsumed under the widespread and common *L. occidentalis*. If no significant differences were shown to exist between diagnostic portions of the *L. occidentalis* and *L. masonii* genomes, then *L. masonii* should be subsumed within *L. occidentalis*, and continued protection under the California Endangered Species Act for *L. masonii* should be reconsidered.

Fallon (2007) noted that genetic information is being used increasingly to resolve taxonomic issues for protection at the federal level under the U.S. Endangered Species Act of 1973 (ESA). She conducted a review of listing decisions made by the U.S. Fish & Wildlife Service and the National Marine Fisheries of species, subspecies, or distinct population segments (DPSs) proposed for protection under the ESA. Fallon determined that the listing fate of a DPS based upon data from more than one genetic marker resulted in a higher probability of protection than candidate taxon or population segment whose discreteness was determined by a single genetic marker. With the cautionary tale of Fallon's findings in mind, we examined the ITS region of the nuclear genome and, to corroborate our ITS findings, conducted an amplified fragment length polymorphism (AFLP) analysis on a similar suite of taxa.

We chose the ITS region in large part because Hardway et al. (2004) found evidence for particularly rapid evolution in the *Oenanthe* clade that includes *Lilaeopsis* when compared to the rest of the taxa. Sequence divergences in this clade averaged 6–7 times higher (approx. 17%) than between species in *Oenanthe* (approx. 2.8%) or *Cicuta* (approx. 2.4%) (Hardway et al. 2004). AFLP analysis was selected as a secondary marker system based on the increasing popularity of this form of DNA fingerprinting as a complementary system in phylogenetic studies (Holland et al. 2008). Additionally, AFLP fingerprinting offers a reliable, robust, and genomically comprehensive method of genetic analysis for taxa lacking complex nuclear and organellar markers (Vos et al. 1995).

Materials and Methods

Field Collection

Lilaeopsis masonii specimens were collected in the spring of 2007 from locations along the Napa River and in the Sacramento/San Joaquin Delta. *Lilaeopsis occidentalis* was collected from Bodega Head, California, and Mason and Lawrence lakes in Washington State (Fig. 1). Leaf material to be used in DNA extraction was preserved in silica gel at the time of collection. Vouchers were deposited at the herbarium at San Francisco State University (SFSU) (Table 1). Material for *L. brasiliensis* (Glaz.) Affolter and *L. mauritiana* G. Petersen & J. Affolter was obtained from a commercial aquarium supplier (freshwateraquariumplants.com). The dataset is composed of 35 nrDNA ITS sequences representing seven taxa, including three sequences

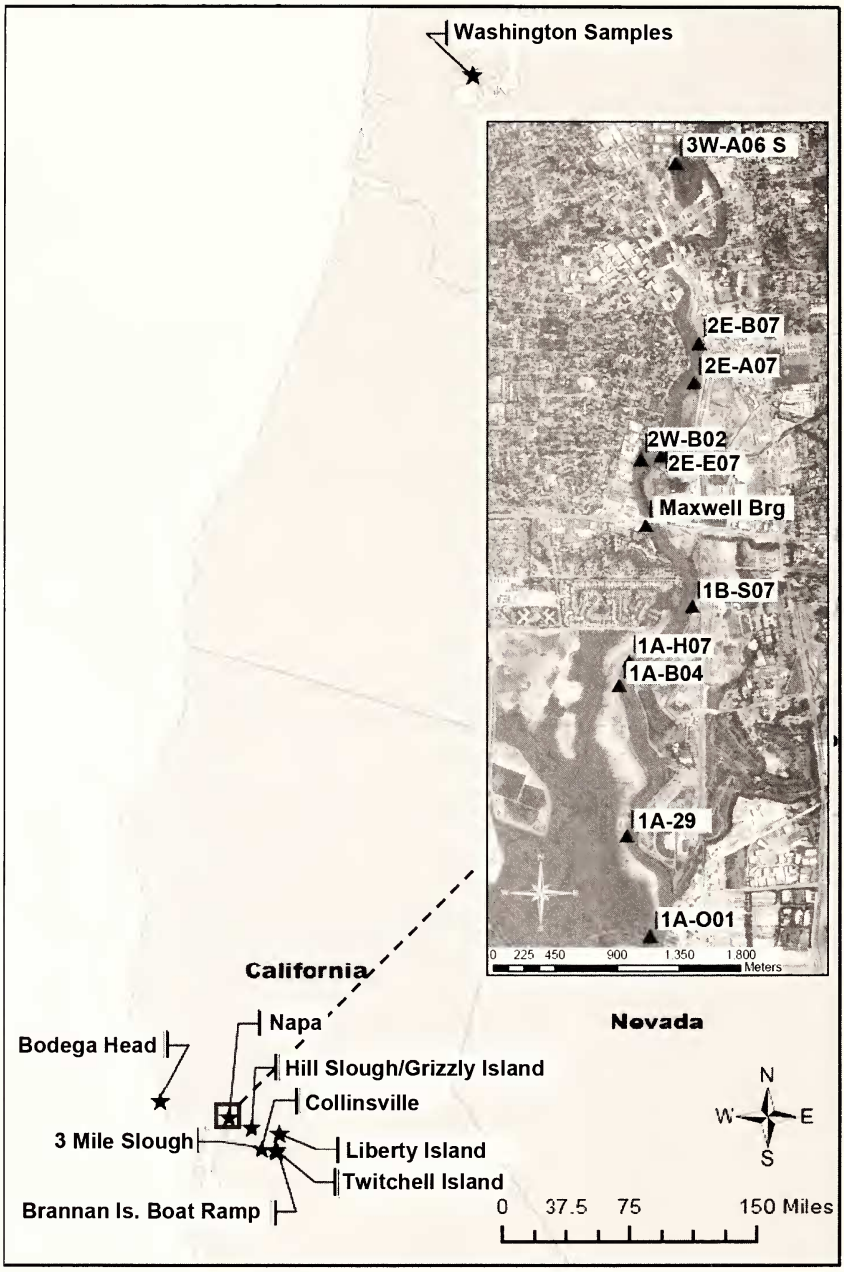

FIG. 1. Map of the geographic locations of *Lilaeopsis masonii* and *L. occidentalis* specimens collected for this study.

from Genbank (*L. carolinensis* J. M. Coulter & Rose, *L. novae-zelandiae* (Gand.) A. W. Hill, and *L. occidentalis*), two specimens from the aquarium trade labeled as *L. brasiliensis* and *L. mauritiana*, and two specimens of *L. schaffneriana* (Schltdl.) J. M. Coulter & Rose subsp. *recurva* (A. W. Hill) Affolter courtesy of the Desert Botanical Garden staff. Comprehensive sampling was conducted for *L. masonii* and *L. occidentalis* as the purpose of this study was to resolve the taxonomic classification for these two species. A detailed systematic study for *Lilaeopsis* is in preparation (S. Downie, Univ. of Illinois, Urbana-Champaign, personal communication).

ITS Methods

DNA from leaf tissue of five of the seven species was extracted using the Qiagen DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA), following the manufacturer's protocol with slight modifications. *Lilaeopsis carolinensis* and *L.*

TABLE 1. COLLECTION INFORMATION AND GENBANK ACCESSION NUMBERS FOR ITS AND AFLP ANALYSES.

Species	Sample location / GenBank reference no.	County, state	UTM	EASTING	NORTHING	Date collected	Voucher location
L. carolinensis	ITS: EF17713	NA	NA	NA	NA	NA	Argentina, cult. Univ. of Mich Botanic Gardens
L. masonii	Napa River 1A-29a	Napa, CA	11S	0562369	4234924	5/6/2007	SFSU
	Napa River 1A-29b	Napa, CA	11S	0562369	4234924	5/8/2007	SFSU
	Napa River 1A-B04	Napa, CA	11S	0562318	4236035	5/9/2007	SFSU
	Napa River 1A-B04 N	Napa, CA	11S	0562303	4236101	5/9/2007	SFSU
	Napa River 1A-H07a	Napa, CA	11S	0562379	4236283	5/8/2007	SFSU
	Napa River 1A-H07b	Napa, CA	11S	0562379	4236283	5/8/2007	SFSU
	Napa River 1A-07/Maxwell Bridge // #HQ634701	Napa, CA	11S	0562510	4237260	5/10/2007	SFSU
	Napa River 1B-S07	Napa, CA	11S	0562839	4236676	4/24/2007	SFSU
	Napa River 2E-A07	Napa, CA	11S	0562852	4238298	4/25/2007	SFSU
	Napa River 2E-B07	Napa, CA	11S	0562888	4238582	4/25/2007	SFSU
	Napa River 2E-E07a	Napa, CA	11S	0562611	4237766	4/24/2007	SFSU
	Napa River 2E-E07b	Napa, CA	11S	0562611	4237766	4/24/2007	SFSU
	Napa River 2W-B02a	Napa, CA	11S	0562463	4237739	4/24/2007	SFSU
	Napa River 2W-B02b	Napa, CA	11S	0562463	4237739	4/24/2007	SFSU
	Napa River 1A-O01/E02	Napa, CA	11S	0562535	4234287	5/6/2007	SFSU
	Napa River - 3W-01 //HQ647239	Napa, CA	11S	562713	4239895	9/13/2007	SFSU
	McAvoy Harbor -001// #HQ647237	Contra Costa, CA	11S	059118	5210846	8/24/2007	SFSU
	3 Mile Slough -002 //# HQ647245	Sacramento, CA	11S	0613933	4218528	8/23/2007	SFSU
	Brannan Island boat ramp -003 //# #HQ647241	Sacramento, CA	11S	0614975	4219420	8/24/2007	SFSU
	Twitchell Island -004/ / #HQ634700	Sacramento, CA	11S	0615455	4216348	8/24/2007	SFSU
	Liberty Island-005 //HQ647238	Solano, CA	11S	0613549	4233900	8/23/2007	SFSU
	Hill Slough/Grizzly Island-006 //# HQ647242	Solano, CA	11S	0585589	4231320	8/26/2007	No voucher
	Collinsville-007 //# HQ647240	Solano, CA	11S	0600939	4214714	8/27/2007	SFSU
L. novae-zelandiae	ITS:AF466278	N/A	N/A	N/A	N/A	N/A	New Zealand, cult. Gitte Petersen
L. occidentalis	Bodega Head // #HQ647236	Marin, CA	11S	0494826	4231320	10/7/2007	SFSU
	Mason Lake, WA //# HQ6472344	Mason, WA	10T	0503357	5241674	7/25/2008	SFSU
	Lake Lawrence, WA //# HQ647243	Thurston, WA	10T	0503357	5188743	7/25/2008	SFSU
	ITS:AY360242	N/A	N/A	N/A	N/A	N/A	USA, Oregon, Douglas Co.
L. schaffneriana	Scotia Canyon, Arizona //# #HQ647231	Cochise, AZ	N/A	Approx. 557009	Approx. 3479631	6/14/1991	SFSU
	Scotia Canyon, Arizona //# HQ647232	Sonoita, AZ	N/A	Approx. 526984	Approx. 3501690	6/17/1992	SFSU
L. brasiliensis	Freshwateraquariumplants.com //# HQ647234	N/A	NA	N/A	N/A	N/A	SFSU
L. mauritiana	Freshwateraquariumplants.com //# HQ647233	N/A	NA	N/A	N/A	N/A	SFSU

TABLE 2. AFLP PRIMER AND ADAPTER SEQUENCES.

Primer	Sequence
Ad1EcoRI	5′ -CTCGTAGACTGCGTACC- 3′
Ad1MseI	5′ -GACGATGAGTCCTGAG- 3′
prampEcoRI	5′ -GACTGCGTACCAATTCA- 3′
prampMseI	5′ -GATGAGTCCTGAGTAAC- 3′
FAM-EcoRI	5′ -GACTGCGTACCAATTCAAC- 3′
HEX-EcoRI	5′ -GACTGCGTACCAATTCACG- 3′
MseI + CAA	5′ -GATGAGTCCTGAGTAACAA- 3′
MseI + CAT	5′ -GATGAGTCCTGAGTAACAT- 3′
MseI + CAG	5′ -GATGAGTCCTGAGTAACAG- 3′

novae-zealandiae were excluded from this analysis due to technical difficulties with the DNA extraction from the leaf material. Dilutions of the genomic DNA extract of 1:10 in ultrapure water were used in PCR reactions. The contiguous ITS1, 5.8S, and ITS2 regions of nuclear ribosomal DNA were PCR-amplified using the primers ITSLEU (Baum et al. 1998) and ITS4 (White et al. 1990) in final reaction volumes of 25 µl. Positive amplifications were purified using the MO BIO UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Inc., Solana Beach, CA). Internal primers ITS2 and ITS3 (White et al. 1990) were used in addition to ITSLEU and ITS4 in cycle-sequencing reactions in order to extend fragments and clarify ambiguities. Fragments were sequenced with the BigDye 3.1 kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocols, and visualized using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were manually aligned using Sequencher 3.1.1 (GeneCodes Corp., Ann Arbor, MI) and MacClade 4.04 (Maddison and Maddison 2001).

AFLP Methods

AFLP fingerprinting was conducted following a modified protocol based on the methods described by Vos et al. (1995). DNA extracts prepared for ITS analysis also were used for this study undiluted. Approximate DNA concentrations for all samples were estimated to contain a range of concentrations from 10 ng/µl to 50 ng/µl using an ethidium dot test. DNA template of each sample was digested using the infrequent endonuclease cutter *Eco*R1 and the frequent endonuclease cutter *Mse*I. Immediately following digestion, the entire digestion reaction was combined with an equal volume of ligation mix.

The resulting fragmented DNA template containing "sticky ends" was diluted five-fold and subsequently amplified by PCR using a pre-selective primer mix. This step effectively reduces the number of possible fragments by approximately 1/16th (Meudt and Clarke 2007). The pre-selective reaction condition consisted of 30 cycles of 94° for 30 sec, 56° for 1 min, and 72° for 1 min.

Three combinations of selective primer sets were used to produce a final AFLP fingerprint for each sample (Table 2). Each set of selective primers consisted of a primer region matching the known adapter sequence, as well as three selective nucleotides on the 3′ end of the *Mse*I primer and three selective nucleotides plus a florescent label on the 3′ end the of *Eco*RI primer. Template for the pre-selective PCR was diluted 6-fold and then combined with a master mix containing one set of selective PCR primers. A step-down PCR was used to amplify the selective fragments in a program consisting of 13 cycles of 94° for 30 sec, 65° for 30 sec (−0.7° per cycle), and 72° for 1 min, followed by 24 cycles of 94° for 30 sec, 56° for 30 sec, and 72° for 10 sec.

The final selective PCR product fragments containing a fluorescently labeled *Eco*RI end and unlabeled *Mse*I end were analyzed undiluted using an ABI 3100 genetic analyzer (Applied Biosystems). Initial fragments were sized first using the analysis software GeneScan (Applied Biosystems) and using the program by GeneMarker© (SoftGenetics, State College, PA). After a comparison of fragment calling using both programs, all samples were analyzed using GeneMarker©.

Fragments were recorded for each sample in a data matrix based on a binary system (1 for presence, 0 for absence); a data matrix was developed for each primer combination and then all data was collated into a single data matrix. To test reproducibility of results, twenty percent of all samples selected at random for each primer pair were re-analyzed, starting with the initial DNA extracts. Fragment peaks that were determined to be consistently low (below 300 peak intensity) or unpredictable were dropped from the matrix table.

Data Analysis

Phylogenetic analyses of ITS sequences were conducted using Phylip version 3.68 (Felsentein 2004). All characters were weighted equally,

character state transformations were treated as unordered, and gaps were treated as missing data. Most-parsimonious trees were obtained in Phylip using the "branch-and-bound" method of exact search implemented by the analysis unit DNAPENNY. Bootstrap re-sampling (1000 replicates) was used to assess nodal support (Felsenstein 1985). Most parsimonious trees were generated from a search of 100,000 trees and a final tree was derived using a strict consensus tree method (Felsenstein 2004). Additional tree searches were conducted in greater volumes, up to 1,000,000. However, larger searches produced the same final tree, thus a smaller tree search was selected to reduce run-time during bootstrapping. Several combinations of *Lilaeopsis* species outgroups were explored before selecting *L. novae-zelandiae* as the outgroup. This selection was based on indications of a potential sister group relationship between *L. novae-zelandiae* and *L. occidentalis*, which was supported by ITS phylogenetic analysis of this genus within the Apiaceae tribe described in Downie et al. (2008). Genetic distances to determine branch lengths were calculated in Phylip using the Jukes-Cantor method implemented in DNADIST and a Fitch-Margoliash (FITCH) search.

AFLP phylogenetic analysis was performed using the program Phylip version 3.68 (Felsenstein 2004). A genetic distance matrix was created using the techniques described by Nei and Lei (1979) as implemented by RestDist in Phylip (Felsenstein 2004). The output matrix was then input into NEIGHBOR using the UPGMA method of cluster analysis (Felsenstein 2004). Using this approach, an output tree was constructed by successive clustering using an average-linkage method of clustering. The output file was then plotted as both a rooted and unrooted tree. A search for the most parsimonious trees was implemented first using the branch-and-bound algorithm of DOLPENNY (100,000 trees searched) in Phylip following bootstrap analysis using 100 replicates. A 50% majority-rule consensus tree was then generated to condense the results into a final tree, which is presented here. Previous studies of *Lilaeopsis* using AFLP analysis have not previously been reported. As such, outgroup selection for AFLP parsimony analysis was determined following variable, preliminary analysis replicates. *Lilaeopsis schaffneriana* was selected as this species is the closest geographically to both *L. masonii* and *L. occidentalis*. Further, *L. schaffneriana* also demonstrated sufficient genetic differences to be used as an appropriate outgroup.

To further visualize potential multi-dimensional correlation of AFLP data based on genetic similarities, an additional genetic distance matrix was derived using Manhattan distance (StatisiXL; www.statistixl.com). These data were then analyzed using a principal coordinates analysis (PCA) using the Microsoft Excel© add-in program GenAlEx 6.2 (Peakall and Smouse 2006). To determine whether a measurable degree of genetic dissimilarity among *L. occidentalis* (WA and CA samples) and *L. masonii* (CA samples) could be attributed to geographic distance, an additional test of molecular variance based on geographic origin as measured by Global Position System (GPS) also was tested. A two-way analysis of variance was assessed for collections of *L. occidentalis* and *L. masonii* using an analysis of molecular variance (AMOVA) with GenAlEx 6.2 (Peakall and Smouse 2006). Significance was assessed using 99 permutations.

Results

DNA sequence analysis of the ITS regions ITS1, 5.8S, and ITS2 of nuclear ribosomal DNA, based on a most parsimonious search of 64,631 trees using a branch-and-bound method, revealed no differences between California samples of *L. occidentalis* and *L. masonii* samples, including a GenBank accession for *L. occidentalis* (100 of 100 trees) (Fig. 2). Within the *L. occidentalis**L. masonii* clade, samples collected from Washington clustered separately. Distance values between Washington and California samples were low for single collections from Lawrence Lake and Mason's Lake (0.1 and 0.3%, respectively). However, a second sample from Lawrence Lake exhibited higher distance values (1.5%), which may be due to missing data. Distance values for California samples of *L. masonii* and *L. occidentalis* were 0% across all samples. Comparatively, distance values between the additional species used for this study ranged from 1.2–8%. Distance based analysis of ITS sequences found an identical tree structure as the strict consensus tree inferred from most parsimonious results implemented by DNAPENNY. Bootstrap estimates from 1000 replicate analyses yielded 100% nodal support for all branches. Branch placement and relationship of *Lilaeopsis* species used in this study are consistent with results of a previous ITS phylogenetic analyses by Downie et al. (2008), though that study excluded *L. masonii*.

The three AFLP primer combinations generated 274 unique fragments among 29 samples, with only 21 fragments shared or monomorphic between the five species used in this study. Although a small sample size was used, specifically for *L. brasiliensis, L. schaffneriana*, and *L. mauritiana*, the large number of shared fragments is potentially indicative of low genetic diversity within this genus, which is consistent with species exhibiting high morphologic plasticity (Linhart and Grant 1996). The number of total diagnostic bands from the three markers combined data set

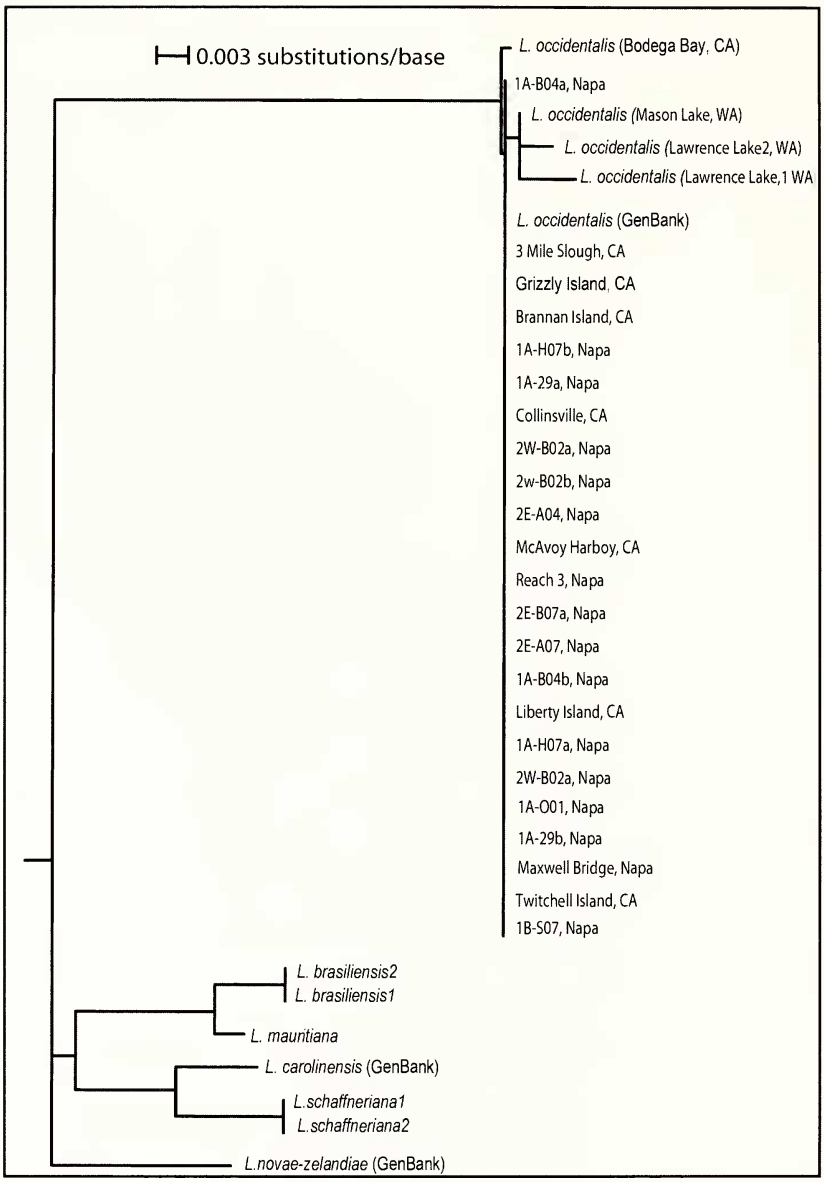

FIG. 2. Strict consensus tree derived from ITS sequence data using the branch and bound method implemented by DOLPENNY. Branch values are Bootstraps. Upper left tree illustrates distance values, branch lengths are proportional to the number of nucleotide substitutions per base.

varied between species, ranging from a low of 98 bands for one sample of *L. schaffneriana*, to a high of 135 bands for the Mason Lake, WA sample of *L. occidentalis*, with a mean number of AFLP bands equaling 123 (SD = 9.5) (Table 3).

Within species genetic distance values (Manhattan distance calculated from the AFLP matrix) ranged from a low of 1.4 for *L. masonii* (Napa River site #1A-H07, large and small forms), to a high of 6.6 for *L. masonii* (Napa site 1A-B04 and Twitchell Island collections). Between species values ranged from a low of 4.1 for *L. occidentalis* (Mason Lake, WA) and *L. masonii* (Napa River #2W-B02), to high of 11.9 for *L. schaffneriana* and *L. occidentalis* (Lake Lawrence, WA collection).

Principal coordinate analysis (PCA) illustrated an overlapping association between samples of *L. occidentalis* and *L. masonii* (Fig. 3), but a clear differentiation between the *L. occidentalis*/*L. masonii* cluster and all other *Lilaeopsis* species examined in this study, i.e., *L. schaffneriana, L. mauritiana*, and *L. brasiliensis*. The small separation observed between the *L. occidentalis* and *L. masonii* data may be attributed to geographic distance. Results of AMOVA analysis derived

TABLE 3. AFLP FRAGMENT NUMBERS FOR EACH SPECIES.

Species	Mean or Total No. of Fragments			
	CAA	CAG	CAT	Total
L. masonii	53.5	46.1	21.9	125.1
L. occidentalis	52.7	50	22.9	128.8
L. schaffneriana subsp. *recurva*	47.5	34.5	16	99.5
L. brasiliensis	52	39	21	114
L. mauritiana	43	38	16	99
All Fragments				123

from grouping samples based on geographic location indicated that approximately 73% of genetic variation was distributed between groups and thus 23% among groups, supporting the conclusion that most observed genetic variation is due to geographic distance ($P = 0.01$).

The UPGMA cluster phenograms provides additional support for the results of the PCA analysis, illustrating a combined grouping of *L. occidentalis* (CA and WA) and *L. masonii* samples (Fig. 4). Within the *L. occidentalis* and *L. masonii* clade, samples collected from Washington clustered separately from samples collected within California, corroborative of results of AMOVA indicating that variation within this clade is due in large part to geographic distance. Samples of *L. brasiliensis* and *L. mauritiana* cluster separately but are sister to the *L. occidentalis*/*L. masonii* clade; *L. schaffneriana* samples also cluster separately but are sister to all other specimens/species used for this study. The most parsimonious tree from the maximum parsimony analysis supports a single *L. occidentalis*/*L. masonii* clade; however, additional resolution within this taxon is less certain of the specific placement of *Lilaeopsis* samples, based on geographic location (Fig. 5).

DISCUSSION

Taxonomic implications. Within the last two decades, the use of genetic techniques to distinguish discrete evolutionary units has become common place in systematic biology. Use of genetic data in the protection of endangered species when morphological (or other character) information is either unreliable or impossible is just one reason why this approach to species identification and delimitation is so important (Avise 2003). Thus, sole reliance on morphological, geographic, reproductive behavior or some combination of non-genetic characters to delimit taxa is no longer defensible when diagnostic genetic information is available and can be readily assessed. In the case of *Lilaeopsis masonii* and *L. occidentalis*, neither ITS sequence nor AFLP fragment length data support the recognition of the *L. masonii* as a distinct evolutionary entity.

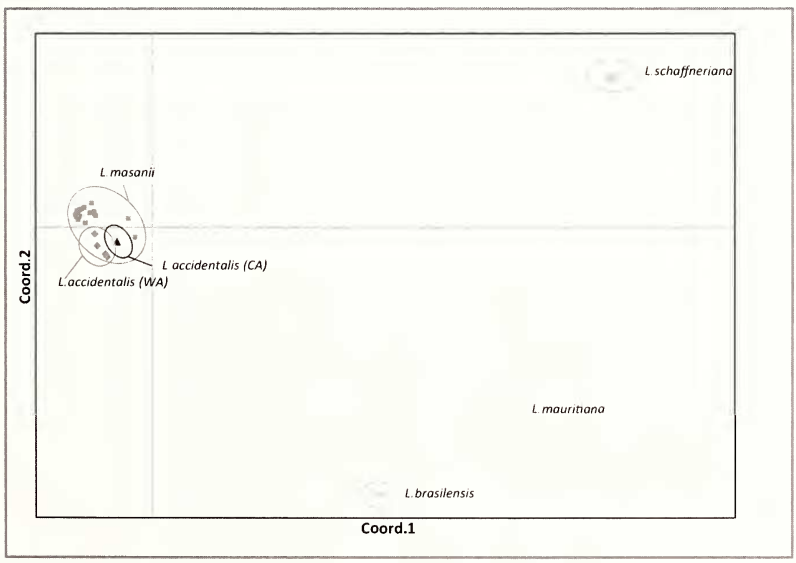

FIG. 3. Principal coordinates analysis (PCA) of AFLP fragment data matrix. Codes for Napa collections, e.g., 1A-H07, indicate different collection locations and dates along the Napa River specific to the Napa River Flood Protection Project.

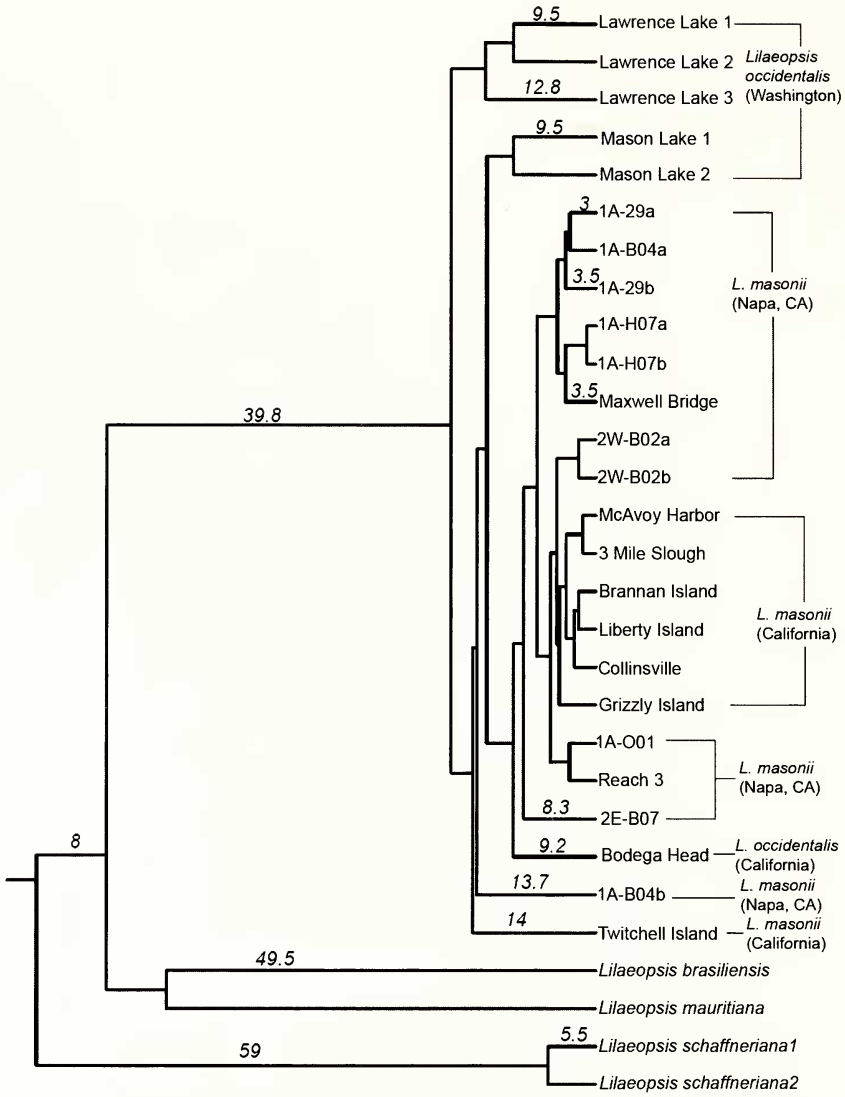

FIG. 4. UPGMA Cluster Phenogram (rooted and unrooted trees) from AFLP data matrix of five species of *Lilaeopsis*. Numbers are distance values. Codes for Napa collections, e.g., 1A-H07, indicate different collection locations and dates along the Napa River specific to the Napa River Flood Protection Project.

The morphological and geographic information to support two distinct taxa is weak, ambiguous, and unreliable at best.

Fallon's (2007) arguments regarding the importance of using genetic information to resolve taxonomic issues for species protection is borne out in our study. While her review focused solely on vertebrates, and on only those species, infraspecific or population segments proposed for listing, not those already listed, our results add further emphasis for use of molecular techniques in conservation efforts. We concur that multiple genetic markers are essential for a thorough assessment of taxonomic or population unit (or at any appropriate level) when considering of formal protection. We further suggest that use of best available science such as existing or generating new genetic information is equally valid for the periodic reviews of listed species required of both the federal and state agencies. Further and relevant to *L. masonii*, use of genetic data is likely to be essential during a de-listing review process.

Based upon several lines of evidence, including decades of fieldwork throughout the range of *L. masonii*, observations from the most recent monograph (Affolter 1985), and our molecular genetic analyses, we urge that this rare taxon no longer be recognized as a separate taxonomic entity. Rather, *L. masonii* should be subsumed within the larger, much more widespread, common, and equally variable species, *L. occidentalis*.

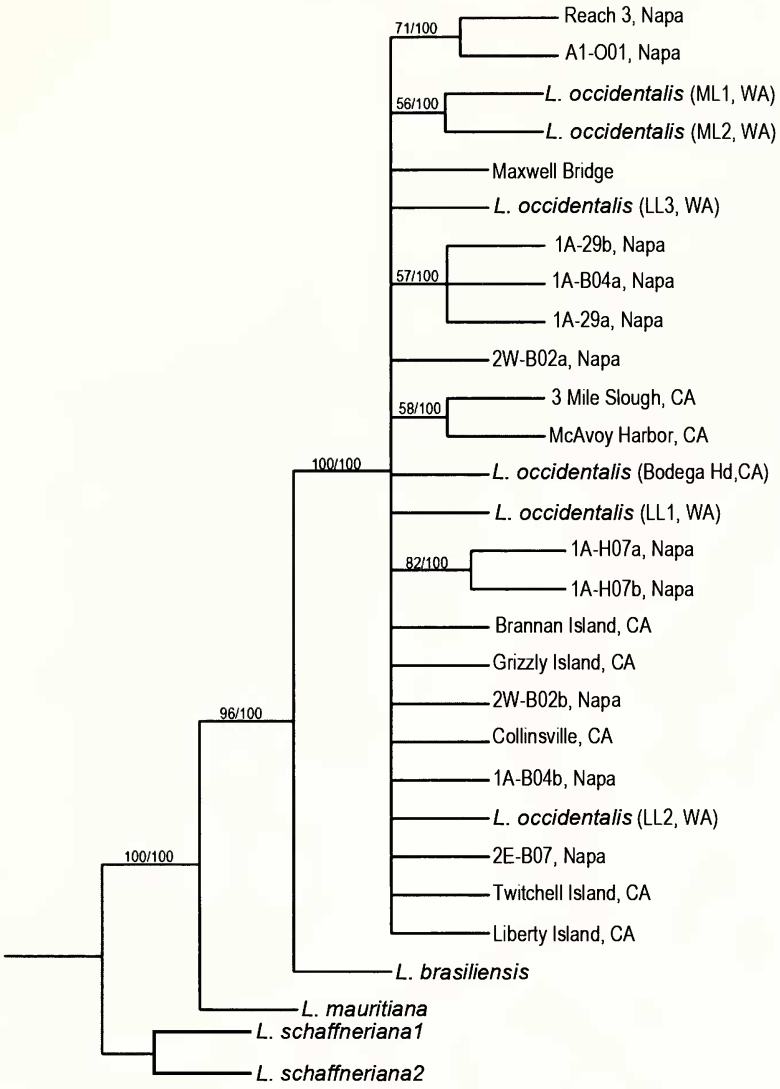

FIG. 5. Most parsimonious tree from AFLP data matrix of five species of *Lilaeopsis*. Codes for Napa collections, e.g., 1A-H07, indicate different collection locations and dates along the Napa River specific to the Napa River Flood Protection Project.

Significant morphological, but limited genetic, variation exists both within and among populations of *Lilaeopsis* throughout the Pacific Coast of North America, from the Queen Charlotte Islands in British Columbia to the inland islands of the Sacramento-San Joaquin Delta in the Great Valley of California. Importantly, this variation does not follow any consistent environmental gradient for either taxon. As such, one intrinsically variable species, not two, of west coast *Lilaeopsis* should be recognized in relevant floras, including those for North America.

Important additional circumstantial support comes from the very wide geographic ranges, some amphitropical, of the great majority of other species of *Lilaeopsis*, including *L. chinensis* Kuntze, *L. carolinensis, L. shaffneriana, L. macloviana* A. W. Hill, and *L. novae-zelandiae*, among others. A large geographic range is not surprising for all these species, given their vigorous vegetative reproduction by easily fragmented rhizomes and their restriction to aquatic habitats, many with bi- or multi-directional flow vectors (e.g., Napa River, Sacramento River, Pacific Ocean).

Field observations suggest a possible explanation for the inter- and intra-populational variation individual ramet size for both western North American *Lilaeopsis*. Periods of rapid spring growth occur during the spring tides and snowmelt from the Sierra Nevada, when temperatures warm sufficiently to encourage an increase in photosynthetic activity. This increased vegetative growth occurs when floodwaters from the

Sacramento, San Joaquin and Napa Rivers are at their height of volume and rate of flow. Thus, the comparatively high kinetic energy of flowing water during the spring run-off, coupled with this species' preference for river banks and shores characterized by high light and open exposure, combine to restrict vegetative growth to a comparatively shorter plant less vulnerable to being dislodged from its habitat. Relatively taller *Lilaeopsis* ramets are invariably found comparatively further from the shoreline in shadier and relatively lower energy microhabitats than comparatively shorter stature ramets. Aquaria enthusiasts who work with various species of *Lilaeopsis* have dubbed the short stature coupled with dense growth phenomenon the "lawn effect" (http://www. freshwateraquariumplants.com).

Conversely, observations of *Lilaeopsis* species submerged in (low energy) water reveal individual leaves grow comparatively longer. Affolter's (1985) greenhouse experiments and observations that demonstrated that for least eight of the 13 species *Lilaeopsis* studied (including *L. occidentalis*), material grown in submerged pots had larger and wider leaves, more septae, and wider rhizome diameter. In his monograph of the thirteen species known in 1985, increased periods of inundation result in a suite of morphological changes, including an increase in leaf length and increases in both peduncle and pedicel lengths (Affolter 1985). Lastly, the rejection of leaf length as a key diagnostic character distinguishing two otherwise very similar taxa has precedent in Affolter's lumping of all Andean, Fuegian, and Patagonian material into a single species, *L. macloviana*, synonomizing thirteen previously described taxa.

Regulatory implications. Neither CESA nor the federal ESA, as amended, protects any vascular plant distinct population segment as does the ESA for specific vertebrate populations. While an argument can be made that this is a form of taxon chauvinism, plant species are not protected below the infraspecific level. Such a comparison is important, because some vertebrate species that were listed relatively soon after the ESA was passed have since been determined not genetically distinct from common widespread relatives, but they continue to be formally protected because of the DPS provisions. For example, the San Francisco garter snake (*Thamnophis sirtalis tetrataenia*), a highly restricted taxon in central coastal California, was determined, through an examination of the clade's mtDNA (Janzen et al. 2002), to be a member of a California clade of the widespread common garter snake. These authors concluded that morphologically based subspecies designations of *T. sirtalis* in western North America were invalid because they did not reflect reciprocal monophyly of mtDNA sequences. Extrapolating Janzen et al.'s (2002) logic to our genetic work with *Lilaeopsis*, the parallel conclusion that the specific designation of *L. masonii* is invalid is compelling. Because neither the CESA nor the ESA include DPS provisions for plant species, *L. masonii* no longer warrants protection as a state "rare" species and the allocation of limited recovery resources. Given the widespread nature of *Lilaeopsis occidentalis* + *L. masonii*, and the large number of projects (both existing and proposed) requiring mitigation and monitoring of the rare *L. masonii*, a timely review of our findings is essential. Conservation dollars are few, and they should be applied to truly rare, threatened, and real discrete species.

Finally, Pavlik (2003) recently examined the role of state- and federally-listed species protecting the ecosystems in which they are found. Of relevance is the notion that some protected species provide a "regulatory umbrella" for other species that are unlisted, but are rare, in decline, or otherwise of conservation concern. *Lilaeopsis masonii* has long served to restrict, prevent, or slow the conversion, degradation or destruction of wetlands throughout the Sacramento-San Joaquin Delta, Suisun Marsh, and San Francisco Bay ecosystems, thereby protecting associated but unlisted species of conservation concern. While a suite of other protected wetland plant taxa exist in these ecosystems (e.g., *Cirsium hydrophilum* Jeps. var. *hydrophilum* [Suisun thistle], *Cordylanthus mollis* A. Gray ssp. *mollis* [soft bird's beak], etc.), there are many more that are rare, in decline, and not listed (e.g., *Cicuta maculata* Lam. var. *bolanderi* (S. Watson) G. A. Mulligan [Bolander's water hemlock], *Plantago elongate* Pursh [slender plantain], *Lycopus asper* Greene) (see Baye et al. 2000). Thus we acknowledge that a delisting of Mason's lilaeopsis may further expedite wetland habitat loss in central California. Nonetheless, conservation in the twenty-first century demands the use of best available science, despite the unintended consequences that may occur. Ultimately, government agencies charged with the protection of our biodiversity must redouble their efforts to embrace new scientific results that affect listed species, commit to diligent review of listed and candidate species, and disseminate accurate and up-to-date information. Similarly, conservationists should redouble their efforts to provide the best available science for decision-making. The time to embrace current molecular genetic techniques in routine conservation decision-making has come.

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